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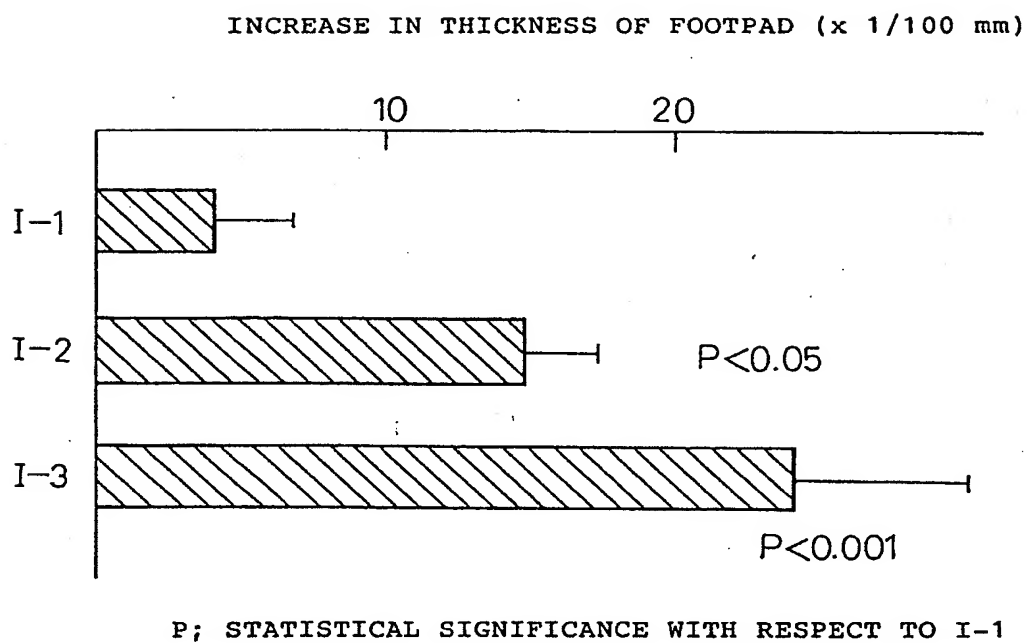
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54 **LIPOSOME HAVING OLIGOSACCHARIDE ON THE SURFACE.**

57 A liposome as an adjuvant which is effective for somatic immunization and reduced in toxicity and antigenicity and can be administered to humans. The liposome comprises 2-11 saccharide residues and has on its surface an oligosaccharide which can combine with a lectin originating in an antigen-presenting cell, and a vaccine is prepared by enclosing an antigen in the liposome.

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Fig. 1



TECHNICAL FIELD

The present invention relates to a method of producing a liposome preparation capable of efficiently inducing cellular immunity, and the liposome may be widely used as an adjuvant (immunostimulating aid) for vaccines and immunotherapeutic agents.

BACKGROUND ART

Because antigens in vaccines and immunotherapeutic agents generally do not elicit an effective immune response by themselves, adjuvants are used as aids to increase immunogenicity. Many substances and preparations with an adjuvant action have been reported as a result of research, but most of these have not been put into practical use due to their strong toxicity, except for aluminum phosphate adjuvants and alum adjuvants, consisting mainly of aluminum hydroxide, being suitable for use in humans.

As an alternative method, there is described in Japanese Unexamined Patent Publication No. 2-188532 a liposome vaccine prepared by reconstructing an antigen-presenting glycoprotein into a liposome. Immunity is largely classified into humoral immunity and cellular immunity, and although alum adjuvants are capable of inducing humoral immunity in a relatively efficient manner, they are not very effective at inducing cellular immunity. Yet in recent years it has gradually become clear that the role of cellular immunity is very important in treatment of persistently infectious viral diseases such as AIDS (J. Salk et al., Science 260, 1270-1271, 1993; M. Sugimoto, K. Ohishi and Y. Ikawa, Immunol. Today 14, 190 - 191, 1993).

Consequently, there has emerged a need to develop adjuvants capable of inducing strong cellular immunity. Liposomes coated with high-molecular polysaccharides such as mannan are reported to have strong cellular immunity-inducing properties (Y. Noguchi et al., J. Immunol., 143, 3737 - 3742, 1989). Also, W092/04887 describes liposomes coated with mannose-containing polysaccharides. However, mannan is a mixture of polymannoses of differing sizes, and it is known to exhibit a strong toxicity in the body (Mikami, K. et al., Summary of 15th Carbohydrate Symposium, 43 - 44, 7/29/93, 30 days, Sendai), and is thus not suitable for use as a drug.

In other words, mannan, being a large polysaccharide consisting of 50 to 100 mannose residues, is also non-uniform in terms of molecular weight, and little is known about its structure, including saccharide bond forms. This polysaccharide produces antibodies when inoculated into animals (it has antigenicity), and as mentioned above it is known to be highly toxic.

DISCLOSURE OF THE INVENTION

The present invention, therefore, is aimed at providing a liposome to be used for humans, which has effective adjuvant activity to induce cellular immunity and which has low toxicity and antigenicity.

As a result of much research intended to overcome the above-mentioned problems, the present inventors have found that liposomes which have oligosaccharide on their surface which consists of 2 to 11 saccharide residues and binds to lectin of antigen presenting cells has effective adjuvant activity to induce cellular immunity and also has low toxicity and antigenicity, to allow its use for humans.

Consequently, the present invention provides liposomes which have oligosaccharide on their surface which consists of 2 to 11 saccharide residues and binds to lectin of antigen-presenting cells.

The present invention further provides a vaccine prepared by encapsulating an antigen in the above-mentioned liposomes.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a graph showing the results of Experiment I.

Fig. 2 is a graph showing the results of Experiment II.

Fig. 3 is a graph showing the results of Experiment III.

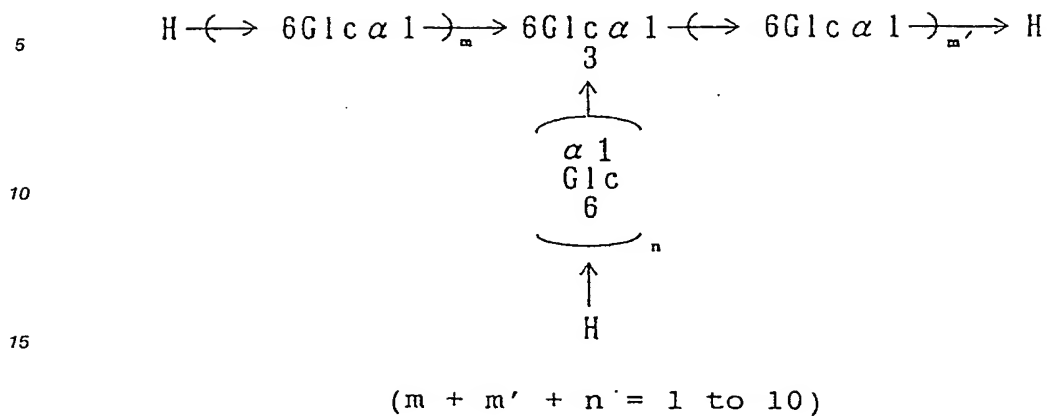
Fig. 4 is a graph showing the results of Experiment IV.

In the figures, the shaded bars indicate average arithmetic means, and the solid lines indicate standard error.

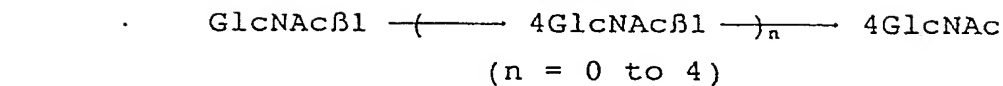
DETAILED DESCRIPTION OF THE INVENTION

The liposomes of the present invention have effective adjuvant activity to induce cellular immunity while both their toxicity and own antigenicity are considered to be low; consequently they may be used as an

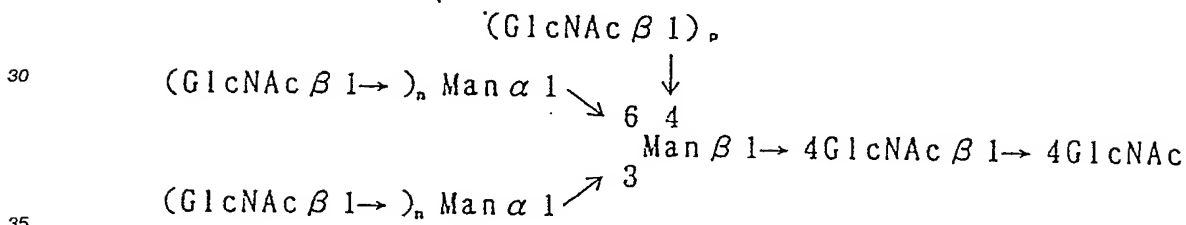
(Compound 3)



(Compound 4-1)



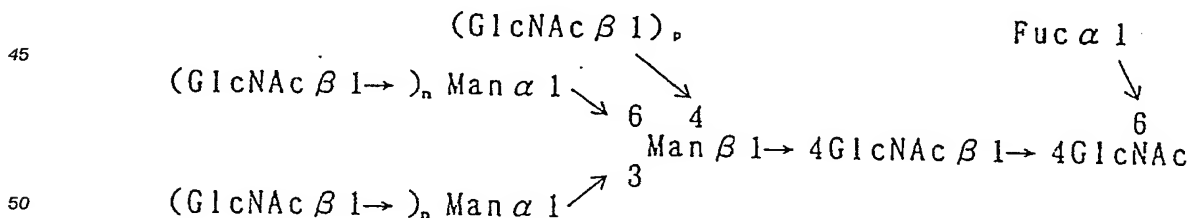
(Compound 4-2)



wherein p is 0 or 1 and each n is independently 0 to 3. One or both of the two GlcNAc residues shown as 4GlcNAc β 1 \rightarrow 4GlcNAc at the right of the formula may either be present or absent independently. Also, any of the GlcNAc residues shown as (GlcNAc β 1 \rightarrow)_n may be glycoside-linked to any free hydroxyl group of the

40 Man residue present the right side of the GlcNAc,

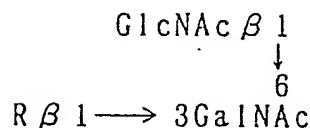
(Compound 4-3)



wherein p is 0 or 1 and each n is independently 0 to 3. Also, any of the GlcNAc residues shown as (GlcNAc β 1 \rightarrow)_n may be glycoside-linked to any open hydroxyl group of the Man residue present the right

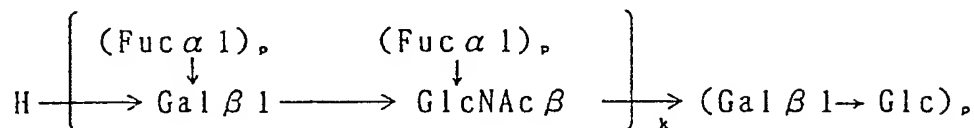
55 side of the GluNAc.

(Compound 4-4)



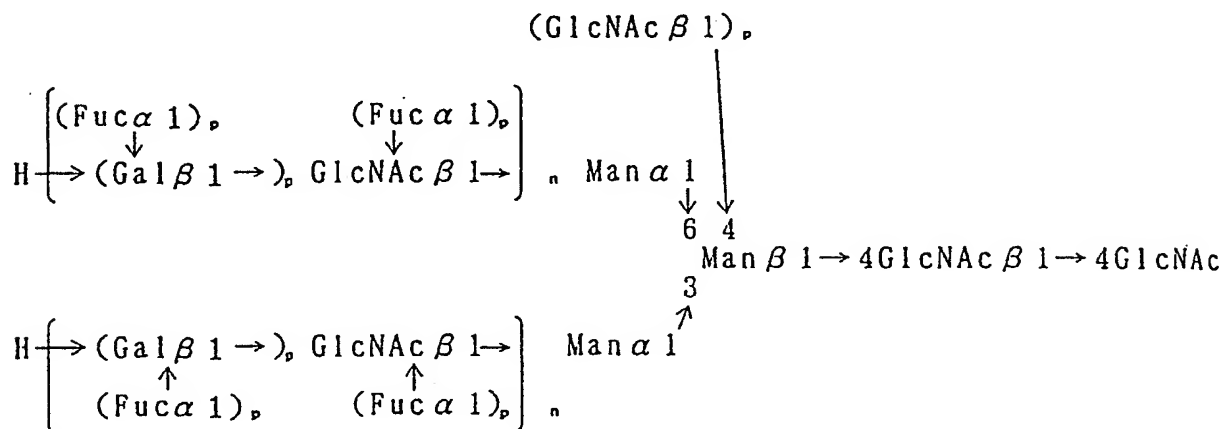
wherein R is H, GlcNAc or (GlcNAc β 1 \rightarrow 6)_p(GlcNAc β 1 \rightarrow 3)_pGal, wherein p is 0 or 1.

(Compound 5-1)



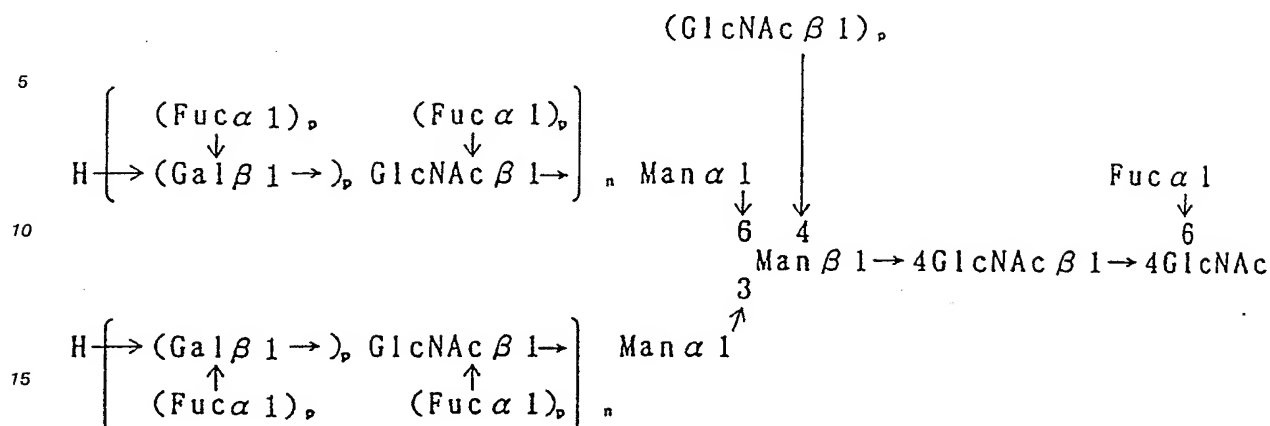
wherein k is 1 to 5 and each p is independently 0 or 1. Those without numbers at the ends of the arrows may be glycoside-linked to any free hydroxyl group.

(Compound 5-2)



wherein each p is independently 0 or 1 and each n is independently 0 to 3. Those saccharides without numbers at the ends of the arrows may be glycoside-linked to any free hydroxyl group. Also, one or both of the two GlcNAc residues shown as 4GlcNAc β 1 \rightarrow 4GlcNAc at the right of the formula may be present or absent independently.

(Compound 5-3)



where each p is independently 0 or 1 and each n is independently 0 to 3. Those without numbers at the ends of the arrows may be glycoside-linked to any free hydroxyl group. Also, one or both of the two GlcNAc residues shown as 4GlcNAc β 1 - 4GlcNAc at the right of the formula may be present or absent independently.

All of the above-mentioned oligosaccharides have one reducing terminal aldehyde group. Therefore, the aldehyde group may be used as the means for introducing the oligosaccharide on the surface of the liposome. That is, a Schiff base may be formed between this aldehyde and the amino group-containing lipid, and the oligosaccharide and the lipid may then be linked by reduction of the Schiff base, preferably chemical reduction such as reduction with NaBH_3CN , using a common method (Mizuochi Tsuguo, Carbohydrate Engineering, pp.224 - 232, Industrial Board of Investigation Biotechnology Information Center, 1992).

The above-mentioned amino group-containing lipid is preferably an amino group-containing phospholipid, and for example there may be used a phosphatidylethanolamine such as dipalmitoyl phosphatidylethanolamine (DPPE), distearoyl phosphatidylethanolamine (DSPE), or the like.

According to the present invention, the oligosaccharide/lipid linked product obtained in the manner described above will sometimes be referred to as an "artificial glycolipid".

Lipids composing the liposome may be any conventional lipids known to be suitable for composing liposomes, used singly or in combination. For example, natural lipids obtained from egg yolk, soybean or other animals or vegetables, or such lipids which have been modified by lowering the degree of unsaturation by hydrogen addition, or chemically synthesized products, may be used. Specific examples include sterols such as cholesterol (Chol); phosphatidylethanolamines such as dipalmitoyl phosphatidylethanolamine (DPPE) and distearoyl phosphatidylethanolamine (DSPE); phosphatidylcholines such as dipalmitoyl phosphatidylcholine (DPPC) and distearoyl phosphatidylcholine (DSPC); phosphatidylserines such as dipalmitoyl phosphatidylserine (DPPS) and distearoyl phosphatidylserine (DSPS); phosphatidic acids such as dipalmitoyl phosphatidic acid (DPPA) and distearoyl phosphatidic acid; etc.

The liposomes are prepared using a per se known method (D.W. Deamer, P.S. Uster, "Liposome" ed. by M.J. Ostro, Marcel Dekker Inc., N.Y. Basel, 1983, p.27). The vortex method and ultrasonic method are known, but other methods which may be applied include the ethanol infusion method, the ether method and the reverse phase evaporation method, used singly or in combination.

For example, when applying the vortex method or the ultrasonic method, lipid is dissolved in organic solvent such as methanol, ethanol, chloroform or a mixture thereof such as a mixture of methanol and chloroform, after which the organic solvent is evaporated off to obtain a thin lipid layer.

Next, an aliquote of aqueous medium is added to the thin lipid layer and it is subjected to vortex treatment or ultrasonic treatment to form liposomes. A desired antigen or immunogen which is to be the active component of vaccine is combined with the above-mentioned aqueous medium at this time, and for example, it may be dissolved or suspended to encapsulate the antigen or immunogen in the liposome.

Any of, for example, the following two methods may be used to introduce the oligosaccharide onto the surface of the liposome. In cases where the above-mentioned artificial glycolipid is water-soluble and does not dissolve sufficiently in organic solvents, for example when products of linking the above-mentioned M5 and DPPE (M5-DPPE), or RN and DPPE (RN-DPPE) are used, an aqueous solution may be prepared and mixed with the formed liposomes and incubated at, for example, 4°C to room temperature for 24 to 120

hours, for example about 72 hours.

On the other hand, if the artificial glycolipid dissolves in inorganic solvents, the artificial glycolipid may be dissolved in the organic solvent together with the liposome-composing lipid, as described above for the liposome-producing process, and the liposome may then be formed by a conventional method.

The amount of the oligosaccharide with respect to the amount of liposomes will differ depending on the type of the oligosaccharide, the type of antigen to be reconstituted, the combination structure of the liposome, and so on, but in general it is between 5 μg and 500 μg per 1 mg of the lipid composing the liposome.

The liposomes of the present invention may be either of multilayer type (multilamella vesicles) or monolayer type (unilamella vesicles). These may be prepared by common known methods, and either type may be converted into the other type by a conventional method; for example, multilayer-type of liposomes may be converted into monolayer-type of liposomes. The particle size of the liposomes of the present invention is not particularly limited, and if necessary the particle size may be adjusted by a conventional method, such as by filtration through a filter of a given pore size.

The antigen to be reconstituted into the liposomes of the present invention may be any water-soluble antigen. As such antigens the followings may be used for example; protein and peptide antigens, and particularly synthetic protein and peptide antigens, for example proteins, glycoproteins, peptides and glycopeptides prepared by extraction from a separation source, gene recombination or chemical synthesis. Examples of such antigens include coat proteins and core proteins, or partial peptides therefrom, of human immunodeficiency virus (HIV), influenza virus, malaria parasites, tubercule bacilli, and the like.

The proportion of the antigen to the liposome is extremely important, and will differ depending on the type of antigen, the composition and structure of the liposome, etc., but generally it is between 1 μg and 100 μg per 1 mg of the lipid composing liposomes.

Linkage of the oligosaccharide to the surface is proven by adding a lectin corresponding to the saccharide, and investigating the agglutination reaction of the liposome.

Liposomes reconstituted with a model antigen is usable to evaluate the effect of the saccharide, and the antigen is preferably a standard protein with high antigenicity which has been thoroughly documented, such as ovalbumin (OVA). The index of cellular immunity may be a delayed-type hypersensitivity (DTH) response in mice (with T_H1 cells).

As shown in the experiment described below, the objective adjuvant was capable of inducing a DTH response. Consequently, it may be used as an adjuvant for protective vaccines of causative agents in which T_H1 cells play a role, and for immunotherapeutic agents and cancer immunotherapeutic agents.

EXAMPLES

The present invention will now be explained in more detail by way of the following examples and experiments.

Example 1: Preparation of artificial glycolipid

To 2.5 to 5 mg each of $\alpha1\rightarrow3$ linked mannobiose (Man2), mannatriose (Man3) having the structure $\text{Man}\alpha1\rightarrow6$ ($\text{Man}\alpha1\rightarrow3$)Man, M5 (compound 1) and RN (compound 2), 600 μl of distilled water was added, and each mixture was stirred to dissolution to prepare an oligosaccharide solution.

On the other hand, DPPE was dissolved in a mixture of chloroform/methanol (1:1 volume ratio) to a concentration of 5 mg/ml to prepare a DPPE solution. Also, $\text{N}_3\text{BH}_3\text{CN}$ was dissolved in methanol to a concentration of 10 mg/ml to prepare a $\text{N}_3\text{BH}_3\text{CN}$ solution.

To 600 μl of the above-mentioned oligosaccharide solution, 9.4 ml of the DPPE solution and 1 ml of the $\text{N}_3\text{BH}_3\text{CN}$ solution were added, and the mixture was stirred. This reaction mixture was incubated at 60°C for 16 hours to produce an artificial glycolipid. The reaction solution was purified with a silica gel column and a C18 reverse phase column to obtain the purified artificial glycolipid. A commercially available mannan cholesterol product (Dojin Chemical) was also used.

Example 2: Preparation of antigen-encapsulating liposome

A 10-mM DPPC-containing chloroform/methanol (2:1, V/V) solution (hereunder referred to as C/M solution) and a 10-mM cholesterol (Chol)-containing C/M solution were mixed at a proportion of 2:1 (usually, total: 3 ml) and the mixture was added to a 25 ml pear-shaped flask which was connected to an evaporator, and the C/M solution was evaporated off at 40°C under reduced pressure. At this time, in the cases where

the oligosaccharide to be added to coat the surface of the liposome was mannoside (Man2) or mannotriose (Man3), the artificial glycolipids prepared therefrom and the DPPE for comparison were dissolved in chloroform and added at a 1/10 molar ratio with respect to the DPPC.

A thin lipid layer was formed at the bottom of the flask, chloroform was added thereto to dissolve the layer, and then the solvent was again evaporated off. This procedure was repeated 2 to 3 times to result in the formation of a smooth thin lipid layer. The flask was placed in a desiccator for at least one hour to completely remove the solvent, and then distilled water was added and the mixture was subjected to a vortex for hydration. The content was transferred to a test tube and pre-cooled at -80 °C for 20 minutes until frozen, and then subjected to a lyophilization to remove the water.

An aqueous solution containing ovalbumin (OVA) (normally 10 mg/ml) as the model antigen was added, and the mixture was subjected to vortex for hydration, and OVA-reconstituted liposomes were formed. PBS (phosphate-buffered saline) was added to the liposome suspension and centrifuged at 15,000 rpm, and the supernatant was removed. After this procedure was repeated, the precipitate was used as liposomes. This was multilamella vesicle-(multilayer-) type liposomes.

In the cases where the oligosaccharide to coat the surface of liposomes was M5 or RN, or mannan (Mn) (comparison), the artificial glycolipid prepared therefrom was dissolved in PBS to a concentration of 2 - 10 mg/ml, this solution was combined with the liposome prepared above at a volume ratio of 5:1, and the mixture was incubated at room temperature for 3 days to adsorb the oligosaccharide onto the surface of liposomes. By measuring the unadsorbed saccharide it is possible to determine the amount of coated glycolipid. All of liposomes were modified with glycolipids, but for simplicity the glycolipids are referred to only in terms of the saccharides, "M5" or "mannan".

The amounts of OVA, Chol and each saccharide contained in the completed liposomes were assayed in the following manner. For OVA, the liposome was dissolved in the surfactant sodium dodecyl sulfate, and the solution was subjected to separation by SDS-PAGE electrophoresis and dyeing with Coomassie brilliant blue (CBB). The degree of dyeing was quantified by densitometry, and the amount of protein was calculated by comparison with a standard sample of OVA. For Chol, the assay was made using a clinical diagnosis kit (Wako Junyaku Kogyo, K.K.), by the cholesterol oxidase/p-chlorophenol method. The saccharide content was assayed by the anthrone-sulfuric acid method.

By following the above procedures, liposomes containing OVA, Chol and glycolipid (dosage per mouse) as listed in Table 1 were prepared.

Table 1

Experiment	Liposome	OVA (μg)	Chol (μg)	Glycolipid (μg)
I	1	5.0	80.2	0
	2	5.0	80.2	166.7 (M5)
	3	5.0	80.2	237.5 (Mn)
II	1	12.3	70	0
	2	12.3	70	185.8 (M5)
	3	12.3	70	3.1 (RN)
	4	12.3	70	68.0 (Mn)
III	1	6.0	60	DPPE
	2	5.5	60	Man2-DPPE
	3	10.3	60	Man3-DPPE
Notes: (1) Values (μg) are amounts per mouse				
(2) The amounts of glycolipid in Experiment III are a 1/10 molar ratio with respect to DPPC.				

Experiments: Induction of a delayed-type hypersensitivity (DTH) response in mice

The above liposomes were inoculated to Balb/c mice (female, 6 weeks old), each group consisting of 5 mice, and the cellular immunity-inducing ability was evaluated by a DTH footpad swelling response. Each of the liposomes listed above were suspended in 0.2 ml PBS, and 0.1 ml thereof was subcutaneously inoculated at each of two places on the dorsal side of mice. At one week after inoculation, 40 μg OVA/22 μg alum/25 μl PBS was injected into the left footpad, and 22 μg alum/25 μl PBS into the right footpad as a

control, and at 24 hours thereafter, the thickness of the left and right footpads was measured. The difference between the thickness of the right and left footpads was used as the index for a specific DTH response.

The results of Experiment I are shown in Fig. 1. The liposomes coated with M5 induced a statistically significantly stronger DTH response than the non-coated ones. Mn had a similar augmenting effect, the degree of which was about the same as M5. That is, there was no statistically significant difference between M5 and Mn groups.

The results of Experiment II are shown in Fig. 2. As expected, the liposomes coated with M5 or Mn induced a statistically significantly stronger DTH response than those not coated with saccharide. RN also had an enhancing effect on the DTH reaction which, though weak, was statistically significant. However, there was no significant difference between M5 and Mn.

To summarize the above two experiments, it is clear that M5 and Mn have roughly equal effects in terms of the induction of a DTH response when they were applied to liposomes.

The results of Experiment III are shown in Fig. 3. Adjuvant activity was compared among OVA-reconstituted liposomes containing DPPE (control) and those containing, instead of DPPE, a conjugate of oligosaccharide and DPPE. Of these, only the groups of manno-*biose* (Man2) and manno-*triose* (Man3) induced a statistically significantly stronger DTH response as compared with the control.

No significant activity was found for conjugates of DPPE with mannose, lactose or galactose (data not shown).

From the results described above it is clear that the conjugates of DPPE with oligosaccharide such as oligomannose consisting of at least 2 to 11 saccharide residues have an effect to augment a DTH response-inducing ability of liposomes when the conjugates were added to the liposomes or applied onto the liposomes. Also, since mannan has a similar effect, oligomannoses with longer length may have the same effect.

The results of Experiment IV are shown in Fig. 4. For comparison the effect of conventional alum adjuvant used as a control is shown. Mice were inoculated with the followings: OVA; mannan-coated liposomes without OVA (IV-1) as a control, OVA in saline (IV-2), OVA in conventional alum adjuvant (IV-3), OVA reconstituted into non-coated liposomes (IV-4) or OVA reconstituted into mannan-coated liposomes (IV-5). The doses per mouse were; 12.5 μ g for OVA, 15 μ g for alum and 40 μ g for liposomes in terms of the amount of cholesterol.

In this experiment, no difference was found among the groups inoculated with OVA in saline, with alum adjuvant or reconstituted into non-coated liposomes. Rather, the alum adjuvant-inoculated group showed a tendency to have the lowest DTH-inducing ability among the three groups. Furthermore, the group inoculated with mannan-coated liposomes exhibited the highest immunogenicity. The DTH response of this group was statistically significantly stronger ($p < 0.05$) than that of the alum adjuvant group.

From the results of Figs. 1 to 4, it is concluded that liposomes coated with oligosaccharides such as mannan or oligomannose are superior to alum adjuvants in regard to the ability to induce a DTH response.

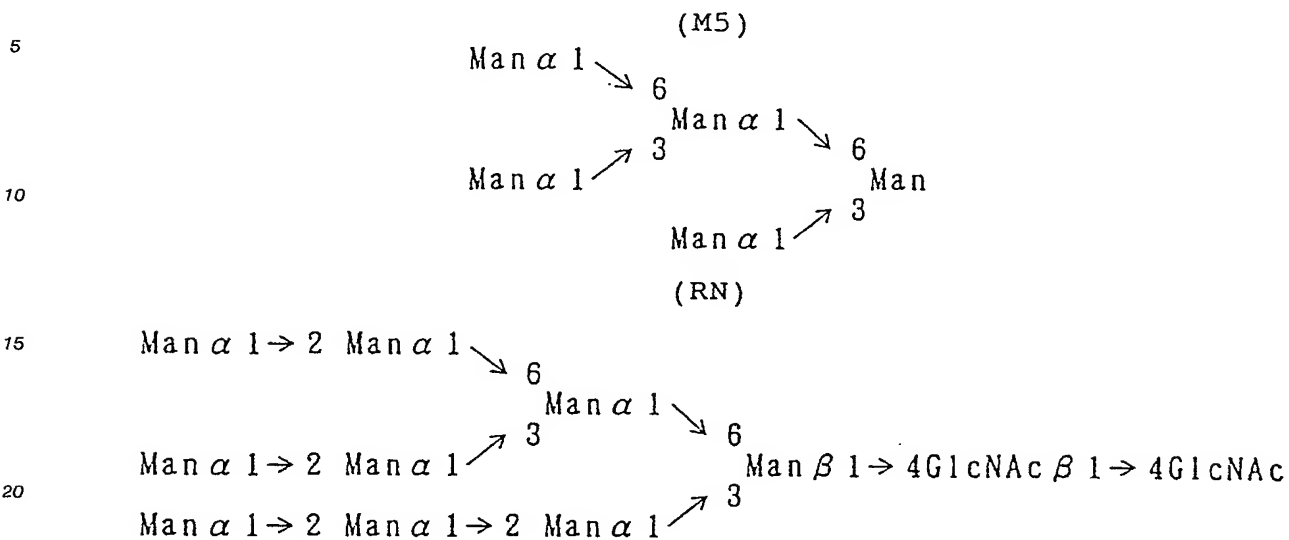
INDUSTRIAL APPLICABILITY

The liposomes of the present invention possess adjuvant activity to induce cellular immunity, with low toxicity and weak or no antigenicity, and may therefore be used as an antigen adjuvant for human vaccines. Particularly powerful vaccines may be obtained by reconstituting an objective antigen or immunogen into the liposomes.

Claims

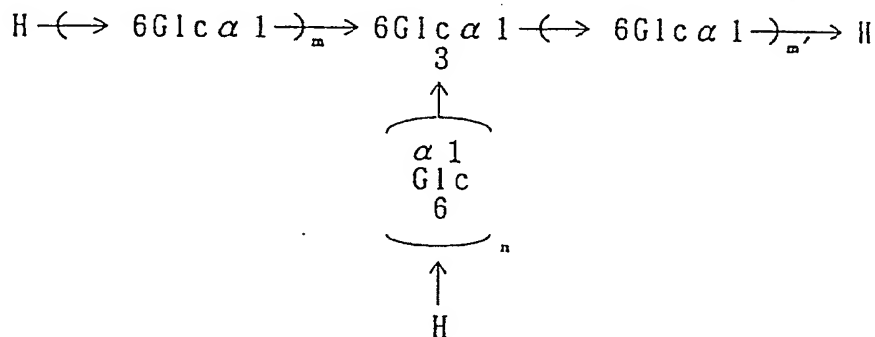
1. Liposomes having oligosaccharide on their surface wherein the oligosaccharide comprises 2 to 11 saccharide residues and binds to lectin of antigen-presenting cells.
2. The liposomes according to Claim 1, wherein the monomers composing said oligosaccharide are one or more monomers selected from the group consisting of D-mannose (D-Man), L-fucose (L-Fuc), D-N-acetylglucosamine (D-GlcNAc), D-glucose (D-Glc), D-galactose (D-Gal), D-N-acetylgalactosamine (D-GalNAc) and D-rhamnose (D-Rha).
3. The liposomes according to Claim 1, wherein said oligosaccharide comprise mannose and is selected from the group consisting of manno-*biose* (Man2), manno-*triose* (Man3), manno-*tetraose* (Man4), manno-*pentaose* (Man5), manno-*hexaose* (Man6) and manno-*heptaose* (Man7).

4. The liposomes according to Claim 1, wherein said oligosaccharide is an oligosaccharide represented by the following structural formula M5 or RN below.



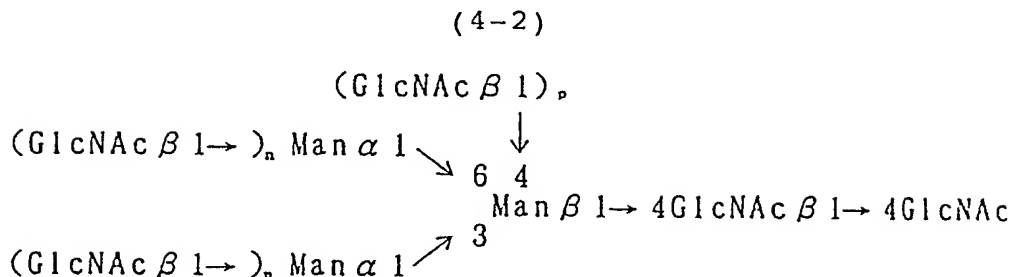
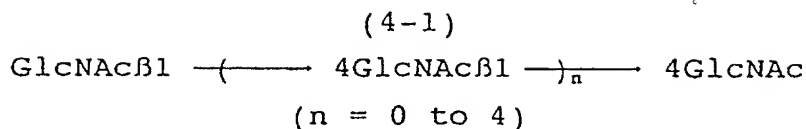
wherein one or both of Man monomers bonded with $\alpha 1 \rightarrow 2$ bond may be present or absent independently.

5. The liposomes according to Claim 1, wherein said oligosaccharide is an oligosaccharide containing glucose and represented by the following formula (3):

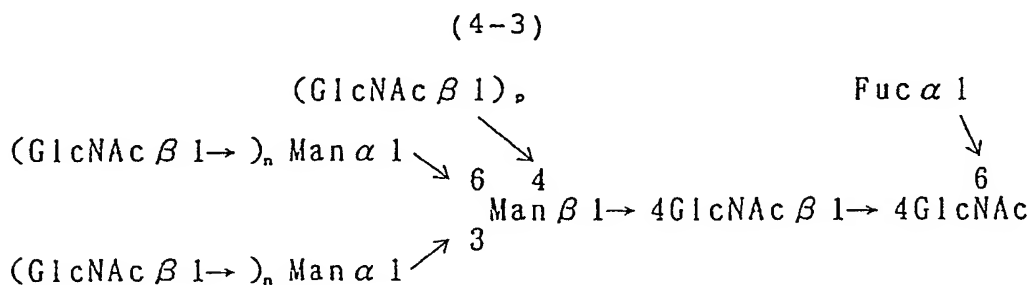


$$(m + m' + n = 1 \text{ to } 10)$$

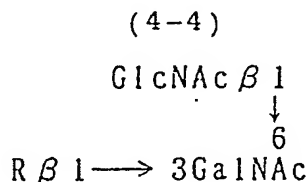
6. The liposomes according to Claim 1, wherein said oligosaccharide is an oligosaccharide containing N-acetylglucosamine and represented by any one of the following formulas (4-1) to (4-4):



wherein p is 0 or 1 and each n is independently 0 to 3, and one or both of the two GlcNAc residues shown as $\text{GlcNAc}\beta 1 \rightarrow \text{GlcNAc}$ at the right of the formula may either be present or absent independently; and any of the GlcNAc residues shown as $(\text{GlcNAc}\beta 1 \rightarrow)_n$ may be glycoside-linked to any free hydroxyl group of the Man residue present the right side of the GlcNAc.

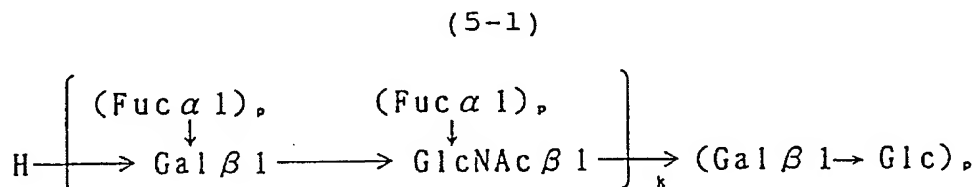


wherein p is 0 or 1 and each n is independently 0 to 3, and any of the GlcNAc residues shown as $(\text{GlcNAc}\beta 1 \rightarrow)_n$ may be glycoside-linked to any free hydroxyl group of the Man residue present the right side of the GlcNAc,

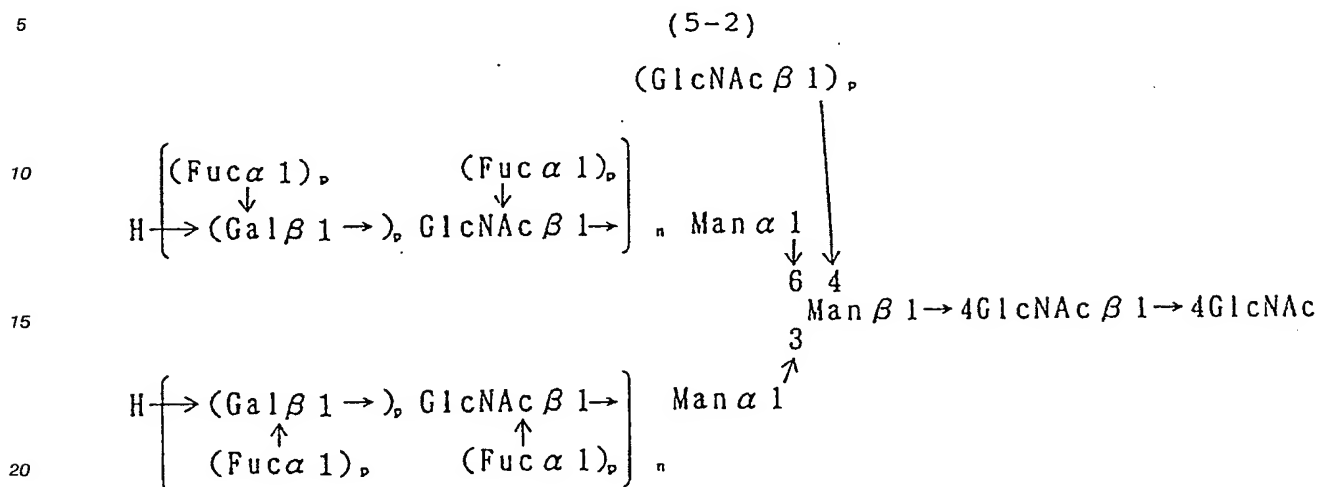


wherein R is H, GlcNAc or $(\text{GlcNAc}\beta 1 \rightarrow 6)_p(\text{GlcNAc}\beta 1 \rightarrow 3)_p\text{Gal}$, and p is 0 or 1.

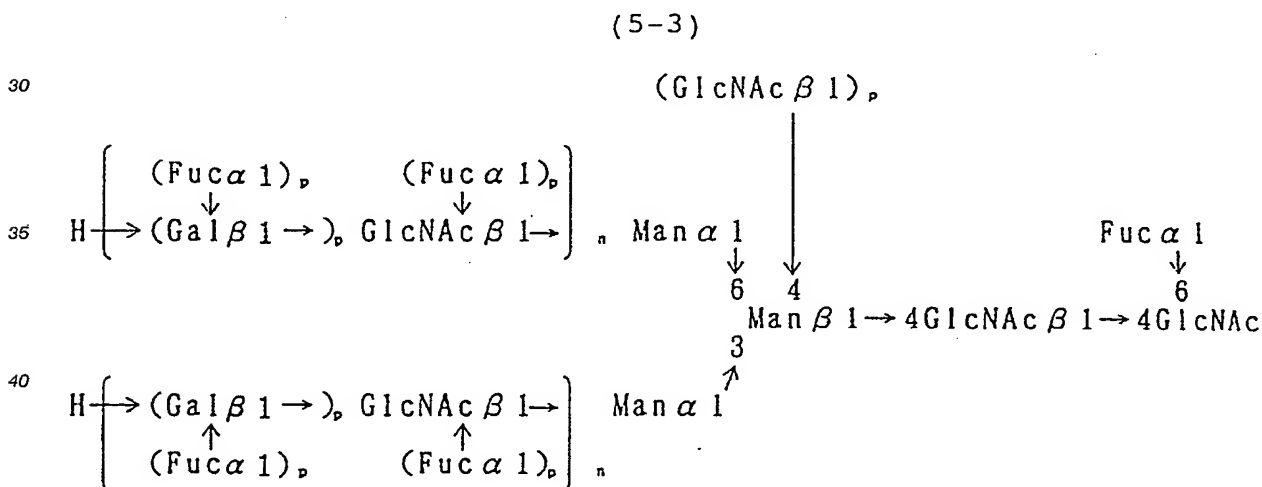
7. The liposomes according to Claim 1, wherein said oligosaccharide is an oligosaccharide containing fucose and represented by any one of the following formulas (5-1), (5-2) or (5-3):



wherein k is 1 to 5 and each p is independently 0 or 1, and those saccharides without numbers at the ends of arrows may be glycoside-linked to any free hydroxyl group,



wherein each p is independently 0 or 1 and each n is independently 0 to 3; those saccharides without numbers at the ends of the arrows may be glycoside-linked to any free hydroxyl group; and one or both of the two GlcNAc residues shown as 4GlcNAc β 1 \rightarrow 4GlcNAc at the right of the formula may be present or absent independently,



wherein each p is independently 0 or 1 and each n is independently 0-3; those saccharides without numbers at the ends of the arrows may be glycoside-linked to any free hydroxyl group; and one or both of the two GlcNAc residues shown as 4GlcNAc β 1 \rightarrow 4GlcNAc at the right of the formula may be present or absent independently.

8. The liposomes according to Claim 1, wherein said liposome is composed by one or more lipids selected from the group consisting of sterols such as cholesterol (Chol); phosphatidylethanolamines such as dipalmitoyl phosphatidylethanolamine (DPPE) and distearoyl phosphatidylethanolamine (DSPE); phosphatidylcholines such as dipalmitoyl phosphatidylcholine (DPPC) and distearoyl phosphatidylcholine (DSPC); phosphatidylserines such as dipalmitoyl phosphatidylserine (DPPS) and distearoyl phosphatidylserine (DSPS); and phosphatidic acids such as dipalmitoyl phosphatidic acid (DPPA) and distearoyl phosphatidic acid.

9. The liposomes according to Claim 1, wherein the lipid composing said liposome is an amino group-containing lipid, and the liposomes and oligosaccharide are linked by reaction between the amino group of the lipid and the aldehyde of said oligosaccharide.

5 10. The liposomes according to Claim 1, wherein said amino group-containing lipid is a phosphatidylethanolamine such as dipalmitoyl phosphatidylethanolamine (DPPE) or distearoyl phosphatidylethanolamine (DSPE).

11. A vaccine prepared by reconstituting an antigen into liposomes according to any of Claims 1 to 10.

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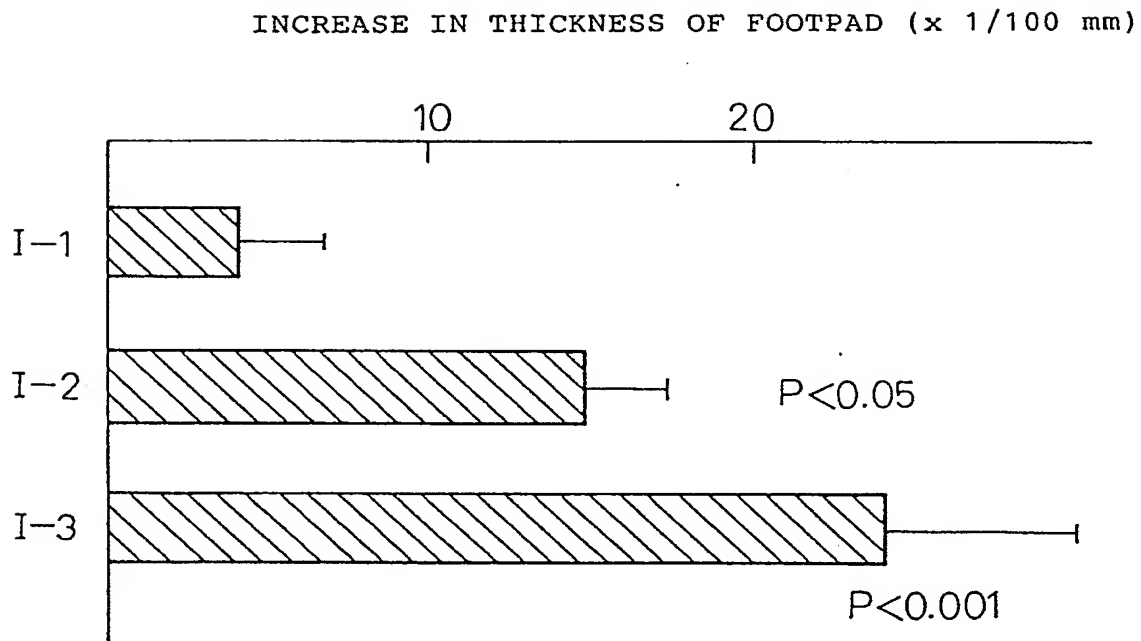
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Fig. 1



P; STATISTICAL SIGNIFICANCE WITH RESPECT TO I-1

Fig. 2

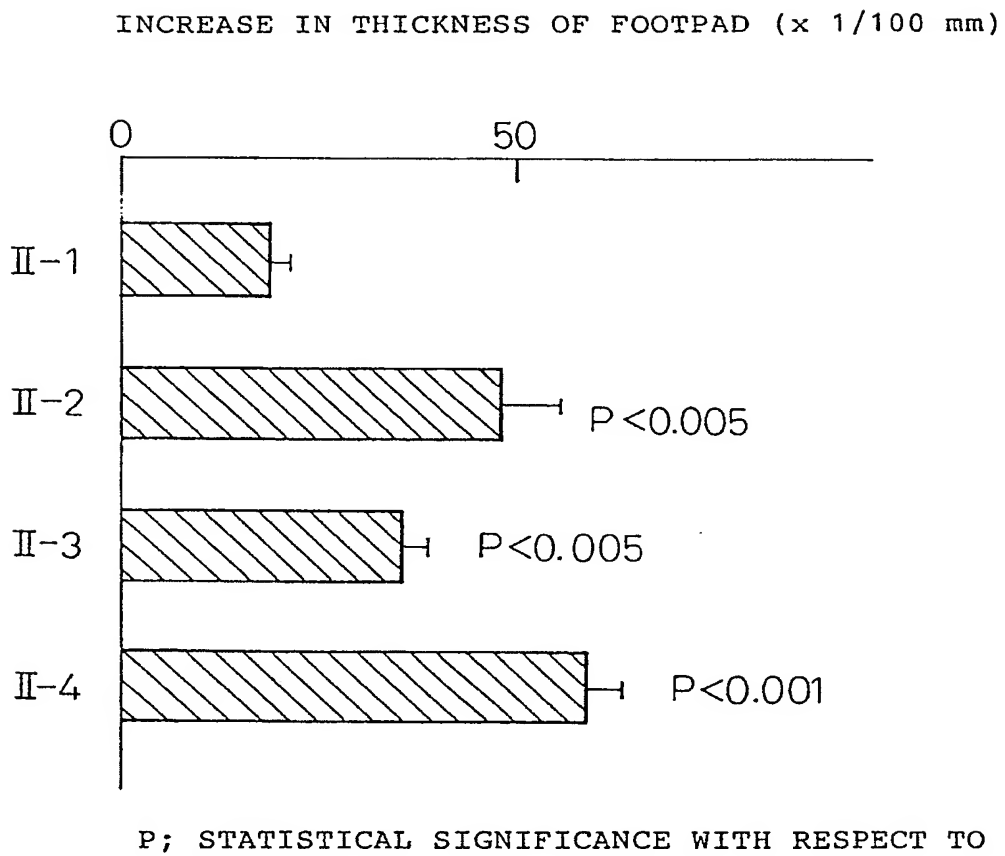
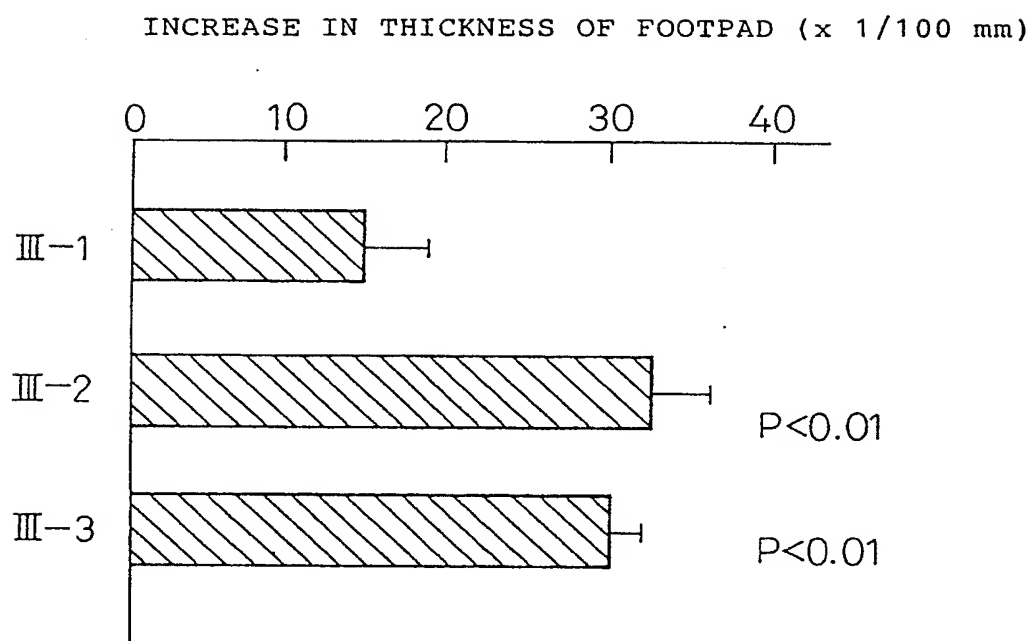
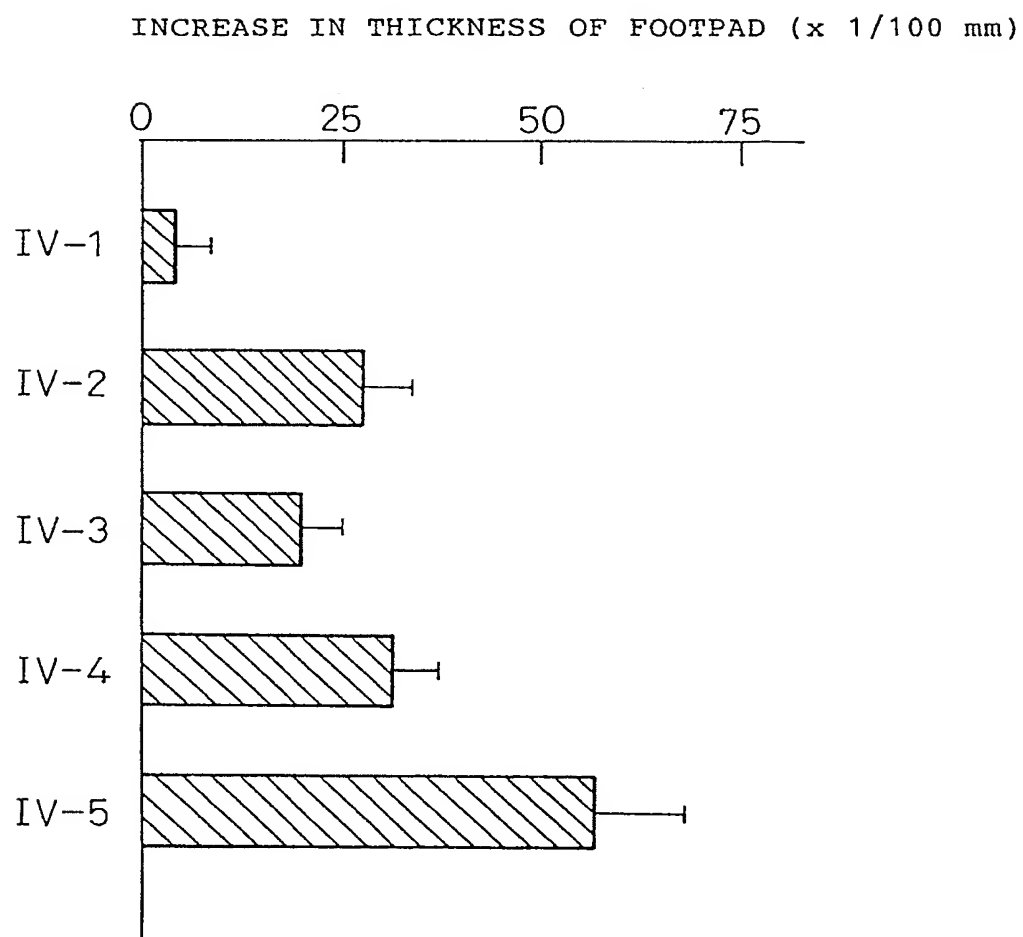


Fig. 3



P; STATISTICAL SIGNIFICANCE WITH RESPECT TO III-1

Fig. 4



INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP94/01828

A. CLASSIFICATION OF SUBJECT MATTER Int. Cl ⁶ A61K47/36 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) Int. Cl ⁶ A61K47/36, A61K39/00 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JP, A, 63-159402 (Rikagaku Kenkyusho), July 2, 1988 (02. 07. 88), Line 5, lower left column, page 1 to line 7, lower left column, page 2, (Family: none)	1-11
A	JP, A, 60-45588 (Rikagaku Kenkyusho), March 12, 1985 (12. 03. 85), Line 5, lower left column to line 3, lower right column, page 1, (Family: none)	1-11
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search December 12, 1994 (12. 12. 94)		Date of mailing of the international search report January 10, 1995 (10. 01. 95)
Name and mailing address of the ISA/ Japanese Patent Office Facsimile No.		Authorized officer Telephone No.

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